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CYTOLOGICAL AND ULTRASTRUCTURAL FEATURES OF INITIATION OF WHEAT MICROSPORE EMBRYOGENESIS

Ari Indrianto[1]

ABSTRACT

Indrianto, A. 2003. Cytological and Ultrastructural Features of Initiation of Wheat Microspore Embryogenesis. Biologi 3 (2) : 65 - 79.

Cytological and ultrastructural features of wheat microspores before and after starvation as well as during the first days of *in vitro* initiation of embryogenesis are described. It is shown that the microspores subjected to stress starvation at 33°C are characterized by the fragmentation of the vacuole, and the formation of cytoplasmic strands that pass through the vacuole and connected a nucleus-containning cytoplasmic pocket with the subcortical cytoplasm. Sporophytic development is started when starved pollen are transfered to non-stress condition, in which 25% of the population of viable embryogenic pollen divided symetrically within 48 hours. Another visible sign of this pathway is mediated by deviation of intine development and proliferation of all cytoplasmic contains.

Keywords : microspore, embryogenesis, stress, ultrastructure, *Triticum aestivum* L.

INTISARI

Indrianto, A. 2003. Gambaran Sitologis dan Struktur Ultra Mikrospora Gandum pada Awal Embriogenesis. Biologi 3 (2) : 65 - 79.

Telah dilakukan pengamatan sitologis dan struktur ultra mikrospora gandum yang diberi praperlakuan rendah nutrisi dan pada awal perkembangan embriogenesis in vitro. Mikrospora yang diberi stres di dalam medium rendah nutrisi pada suhu 33°C memperlihatkan ciri sitologis yang khas, ditandai dengan adanya fragmentasi vakuola dan pembentukan jembatan sitoplasma yang menghubungkan kantong sitoplasma yang mengandung inti dengan sitoplasma di bagian subkortikal. Perkembangan sporofitik diawali oleh adanya perkembangan embrio somatik yang dimulai 48 jam setelah mikrospora dibebaskan dari stress. Perkembangan tersebut ditandai dengan adanya pembelahan simetri mikrospora yang mencapai 25% populasi, terjadinya penyimpangan perkembangan intin dan proliferasi semua isi sitoplasma.

Kata kunci : mikrospora, embriogenesis, stress, ultrastruktur, Triticum aestivum L.

[1] Faculty of Biology, Gadjah Mada University, Yogyakarta

INTRODUCTION

The male gametophyte of flowering plants can be converted from their normal gametophytic mode of development towards the sporophytic development *in vitro* by various stresses applied at plant, organ or microspore level. The resulted haploid plants can diploidize spontaneously or by using diploidizing agents, such as colchicine. These homozygous diploids are the important component for the breeding programs in many important crops, including wheat.

An appropriate developmental stage of the microspore and a specific stress treatment are required in the same time for the induction of embryogenesis. In *Brassica napus* for instance, the late uninucleate and early binucleate stage are competent for this induction (Custers *et al.,* 1994). Heat shock at 32°C for about 8 hours is sufficient to induce embryogenesis (Pechan *et al.,* 1991). In tobacco and wheat the embryogenic microspores are isolated at late microspore stage to premitosis subjected to starvation, and/ or heat shock before culture in the rich medium (Touraev *et al.,* 1996a, 1996b).

Despite of the great progress in inducing embryogenesis from major important crops, the frequency of embryogenesis is still very low; only small fraction of the initial population of cultured micro-spores give rise to embryo formation and haploid plant. The identification of embryogenic state of microspores and their pattern, therefore, is an important issue, which would facilitate the optimization of culture condition to increase the frequency of induced microspores

Light microscopy studies revealed the changes in morphology of stressed microspores. In *Brassica napus* the vacuole becomes fragmented allowing the nucleus to assume a central position within the cell (Zaki and Dickinson, 1990). This morphological feature is also observed in tobacco microspores and mid-bicellular pollen after starvation (Kyo and Harada, 1986; Touraev *et al.,* 1997). These structural changes were claimed to be a typical features of the embryogenic state, since: a). more than 70% microspores in tobacco belong to mentioned type and b). frequency of the induction of embryogenesis in these cultures are more than 60%, which shows indirectly that star-like microspores contribute to embryo formation. However, the light microscopic observations need to be confirmed and substantiated by electron microscopic studies in highly efficient microspore cultures.

Ultrastructural feature of embryogenic and non-embryogenic pollen have been described already in several plant species. Tannin-coated tonoplast were found in microspore of *Datura metel* during *in vitro* culture (Sangwan and Camefort, 1989) and were suggested as

a marker for embryogenic microspores. Sangwan and Sangwan-Norreel (1987) described the development of plastids in non-androgenic and androgenic plants. Proplastid were found to be specific to pollen grains of androgenic plants, while amyloplast were characteristic of the pollen grain of recalcitrant species. Dunwell and Sunderland (1975) observed the appearance of the zone of multivesiculate bodies resembling lyposome and degeneration of plastids in embryogenic pollen in tobacco anther cultures. Garrido *et al.* (1995) reported the lost of nuclear pores in the vegetative nucleus of starved pollen as a prelude to embryogenesis in isolated tobacco microspore culture. Rashid *et al.* (1982) described the formation of fibrillar wall around the pollen cytoplasm and within the intine during embryogenesis in tobacco pollen. The important role of the vegetative cell in the formation of pollen embryo was documented in maize anther cultures (Barnabas *et al.,* 1987). Chen *et al.* (1984) observed an increase in the number of mitochondria, dictyosome, lipid storage and elaboration of the pollen grain intine in barley anther cultures. Dunwell and Sunderland (1976) followed the division of vegetative cell, changes in cytoplasmic contains including an increase in electron density of plastids and mitochondria, deposition of an electron dense materials on the tonoplast of *Datura innoxia* anther cultures. In *Brassica napus*, Zaki and Dickinson (1990) found structural changes in cytoplasmic organisation, including starch synthesis, development of a thick fibrillar wall adjacent to the intine of embryogenic pollen. Telmer *et al.* (1993) observed symmetric division with planar wall of heat-treated microspores of *Brassica napus*. In wheat, the ultrastructure of pollen development is reported in detail by El-Ghazaly and Jensen (1990), Mizelle *et al.* (1989), and Schulz *et al.* (1993), whereas the ultrastructural features of isolated wheat embryogenic microspores have not yet been described.

A critical factor of all these studies is the low frequency of embryogenic microspores which makes the identification of embryogenic microspores using electron microscopy technique very difficult, and the reported features of embryogenic microspores questionable. We have developed a highly efficient system of wheat microspore culture in which almost 75% cells after purification in Percoll gradients divide and form multicellular structures (Indrianto *et al.*, 1999). This system allow us to study the cytological and ultrastructural features of the early events of embryogenesis in wheat microspores.

MATERIALS AND METHODS

Growth of donor plants

Seeds of Austrian winter wheat cultivar Ferdinand were sown in pots (3400 cm^3, 18 cm diameter) with soil, and germinated for one month in a growth chamber at 15°C during the day and 12°C at night, with a day length of 16 h, 60% humidity and light intensity of 800 ME. Germinated seedlings were vernalized at 4°C for two months (10h light, 80% humidity, 800 ME). Finally, the vernalized plantlets were transfered to eliMate chamber where they were grown until tillering under the same conditions for seed germination.

Cold-pretreatment of tillers

Tillers with still ensheated spikes in the late unicellular to premitotic stage were cut and placed upright into water-containing jars. Twenty to 30 tillers in jar were kept for 7 days in the vernalization chamber at 4°C.

Isolation procedures

The cold pretreated tillers were surface-sterilized with an aerosol of 70% ethanol, and spikes were removed from the ensheating leaves. Anthers were isolated and placed on the surface of 1.5 ml of starvation medium B containing 1.49 g/l KCl, 0.12 g/l $MgSO_4$, 0.11 g/l $CaCl_2$, 0.14 g/l KH_2PO_4, 54.7 g/l mannitol, pH 7.0 (Kyo and Harada, 1986) and cultured at 33°C for 4 days. The microspores were isolated by stirring the anthers in medium B with magnetic bar for 2-3 min. at 750 rpm. After three times washing in the medium B by centrifugation for 5 min. at 100 X *g*, the suspension was diluted in 1 ml of medium A2 (Touraev *et al.*, 1996a) at a density of 20.000 microspores per ml and cultured at 25°C in the dark. Medium A2 contains half strength N6-macrosalts (Chu *et al.*, 1990), half stregth B5-micro minerals and vitamins (Gamborg, 1970), 32 g/l Fe-NaEDTA, 10mM MES (2-[N-Morpholino] ethanesulfonic acid), 8.5 mM glutamine and 0.3M maltose as carbohydrate source, pH 6.2. Both B and A2 media were filter sterilized before use. Medium A2 was ovary-conditioned in all cases by placing five ovaries into the 1 ml microspore suspension. The ovaries had been dissected out from the tillers used for microspore cultures 4 days before microspore isolation and were cultured in medium A2 until used.

Cytological observation

To determine the viability and morphology of the cytoplasm the microspores isolated from cold pretreated tillers and microspores isolated from starved anthers were stained with FDA solution, for 10 min. and observed under the fluorescent microscope Leitz - using FITC filter.

The developmental stage of

microspores from freshly harvested tillers (day 0), after stress pretreatments (cold pretreatment of the tillers at 4°C for 7 days plus preculture of anthers for 4 days in medium B at 33°C) and subsequent development in non stress condition (2 days and 6 days in A2 medium at 25°C) were determined in samples stained with DAPI (Vergne *et al.*, 1987). Stained microspores were examined under the fluorescent microscope Leitz - using UV light (filter set A2). A total of at least 300 microspores were evaluated and 5 replications were carried out for every developmental stages.

Electron microscopy

Wheat anthers and microspores isolated from freshly harvested tillers (day 0), after stress pretreatments (cold pretreatment of the tillers at 4°C for 7 days plus preculture of the anthers for 4 days in medium B at 33°C) and after culture in embryogenesis medium for 2 and 6 days were fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 6 h at room temperature. After rinsing in buffer and distilated water, post fixation was carried out in 1% OsO_4 plus 0.8% $K_4Fe(CN)_6$ (3:1) at 4°C overnight. After washing in distilated water, samples were dehydrated in 2,2-dimethoxypropane followed by aceton and embedded in Spurr's low viscosity epoxy resin. Polymerization was carried out at 70°C overnight. Ultrathin section were stained with 1% periodic acid for 10 min., 0.2% thiocarbohydrazid for 15 min., 1% silver proteinate for 10 min., and examined in a Zeiss EM 900 at 50 KV.

Table 1. Percentage of development stage of microspores embryogenesis on the time of induction (0 day), after induction (P-grain, 11 days) and after 2 and 6 days cultured in embryogenesis A2 medium.

Isolated microspores	Uninucleate	Binucleate		Tri nucleate	Multi nucleate	Total
		Asymmetric	Symmetric			
FRESH (0 day)	98,1±0,2	1,8±0,2	-	-	-	134,8±1,6
P-grain (11 days)	84,3±0,6	15,7±0,6	-	-	-	359,2±5,1
In A2 (2 days)	70,1±0,6	4,9±0,3	21,6±0,6	3,01±0,2	-	425,4±8,5
In A2 (6 days)	8,9±0,7	3,8±0,4	17,7±0,6	2,86±0,2	45,6±1,12	286,4±10,3

RESULTS AND DISCUSSION

Cytological and ultrastructural features of fresh, non-stressed wheat microspores.

In wheat, microspores at the late uninucleate to premitosis stage were shown to be competent for the induction of embryogenesis. Cytological analysis after staining with DAPI revealed that 98% of the population consist of uninucleate microspores, and only 2% are binucleate pollen (Table 1.). This data indicated that development of the microspores within the anthers from one spike is synchronous and cytological and ultrastructural analysis can be performed with this material.

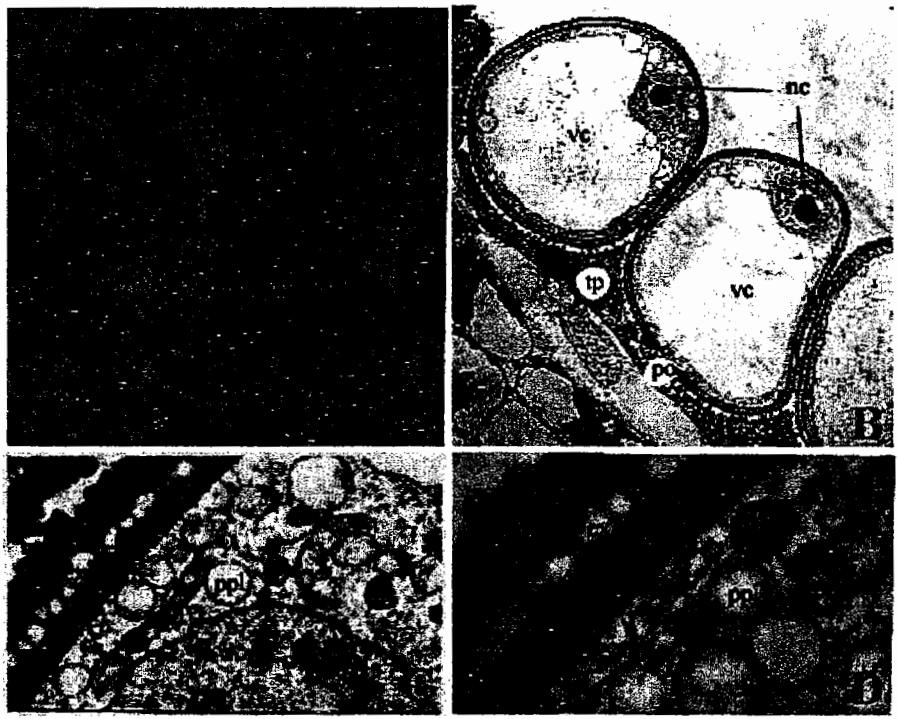
As a member of Poaceae family, the wheat microspore has a single pore oriented towards the tapetum (Fig. 1.B.). At the late uninucleate stage, a big central vacuole occupies most of the lumen of the cell while the nucleus lays close to the microspore wall (Figs. 1.A. and B.). The nucleus is positioned usually opposite to the germ pore (Figs. 1.A. and B.). The cytoplasm is restricted to a thin subcortical layer, it is relatively unorganized, and contains few organelles: small and simple structured proplastids, and mitochondria (Figs. 1.C. and D.), and occasionally dictyosomes. There is no amylogenesis, and starch accumulation is observed. The timing of accumulation of starch is believed to be one of the main determinants of the gametophytic mode of development in different species (Sangwan and Sangwan-Norreel, 1987). In wheat starch accumulation starts at bicellular pollen stage while in snapdragon (*Antirrhinum majus*) and tomato (*Lycopersicon esculentum* L.) the starch was detected very early during pollen development, starting from tetrads and continuing throughout development until mature pollen formation. Based on these phenomenons, Sangwan and Sangwan-Norreel (1987) hypothesized that in all androgenic species, the accumulation of starch starts at the late binucleate stage, whereas in the recalcitrant species accumulation begins much earlier.

The microspore wall is exclusively formed by the ectexine, consisting of a thick tectum, short columellae and a thick foot layer. Both foot layer and tectum are perforated by electron-translucent channels. Intine is not yet developed (Figs. 1.C. and D.). The secretory tapetum becomes compressed (Figs. 1.B.) and starts degenerating.

Cytology and ultrastructure features of stressed wheat microspores

Before isolation of microspores, wheat anthers isolated from cold pretreated tillers were subjected to starvation on mannitol-containing medium B during 4 days at 33°C. The viable embryogenic wheat microspores were sepa-

rated from plasmolysed and dead cells by Percoll density gradient centrifugation (Touraev *et al.*, 1996a). Cytological analysis using DAPI stain showed that majority of microspores (84%) were still uninucleate, while 16% of microspores the nucleus divided asymmetrically (Table 1, P-grain). These data indicate that the

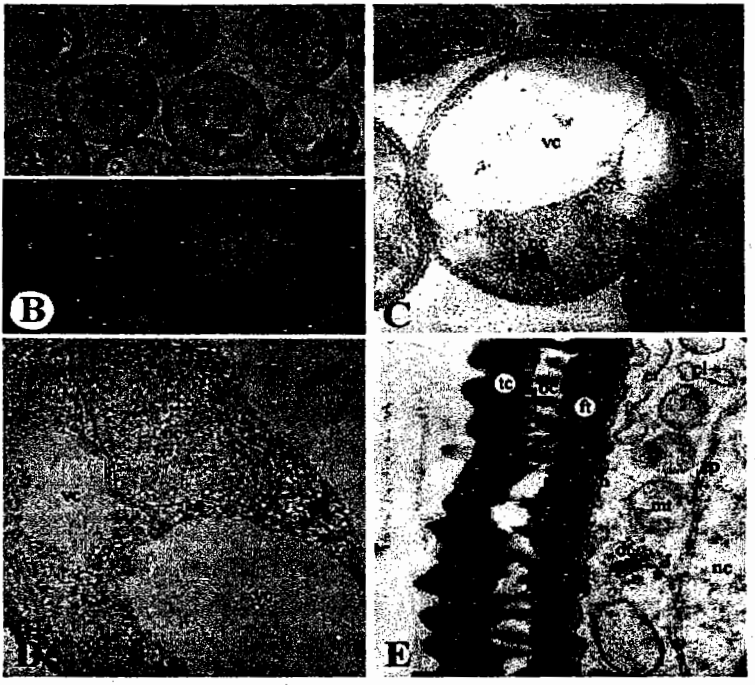


Figures 1A - D: Cytological and ultrastructural features of fresh, non-stressed wheat microspores.

A. Freshly isolated non-stressed microspore at the late uninucleate stage, a big centralvacuole (vc) pushes the nucleus (nc) close to the wall (X 400).

B. Ultrastructural section of the anther containing microspores at the vacuolated stage, A prominent nucleus (nc) is located at the opposite side of the pore (po), tapetum (tp) is compressed (X 1100).

C & D. Section of vacuolated non-stressed microspore. The exine (ex) of microspore wall, proplastids (ppl) and mitochondria (mt) in the cytoplasm are shown (C: X 12.000; D: X 20.000



Figures 2A - E: Cytological and ultrastructural features of stressed wheat embryogenic microspore.

A & B. Fraction of viable stressed microspores after purification by Percoll gradient centrifugation (A, X 200) and staining FDA (B, X 250). The cytoplasmic strands pass through the vacuole, which is fragmented.

C. Section of anthers containing stressed microspore. The cytoplasmic strands pass through the vacuole (vc) and connected to the cytoplasmic pocket (phragmosome) with the nucleus (nc, X 1000). Tapetum is degraded.

D. Cytoplasmic strands containing irregular nucleus (nc) and organells that pass through the vacuole (vc, X 3000).

E. The wall exine consisting of tectum (tc) and foot (ft) layer connected with baculae (bc) or columellae. Both tectum and foot layers perforated by microchannels. Intine wall (in) formation just started, dictyosome (ds), mitochondria (mt), plastids (pl) and endoplasmic reticulum (er) are shown

majority of uninucleate microspores probably are in G1 phase of the cell cycle and remain arrested in this phase during stress pretreatment, whereas the minority which were in G2 phase passed through mitosis during stress pretreatment, indicating that gametophytic arrest had started. Similar data has been obtained in isolated tobacco microspore cultures after starvation and heat shock (Touraev *et al.*, 1997).

The microspores show a dramatic increase in size (Figs. 2.C. vs 1.B.). The nucleus of the uninucleate microspores is irregular in shape (Fig. 2.D.) with convoluted membrane, contains many pores (Fig. 2.E.) and remains in the original position close to the microspore wall (Fig. 2.C.).

In the enlarged microspores the thickness of both tectum and foot layer decreases and the micro channels in both layers are more elaborated (Figs. 2.E. vs 1.D.). Mizelle *et al.* (1989) observed that during normal gametophytic development the maximum thickness of both layers occurs at the stage of vacuolated microspore. This means that during the stress pretreatment deposition of the exine wall material was arrested, probably due to the precous degeneration of tapetum (Fig. 2.C.), which is playing the significant role in the formation of exine. The presence of intine wall is also a characteristic of the normal gametophytic development. Mizelle *et al.* (1989) reported that the deposition of intine wall starts immediately after the first pollen mitosis in wheat. In stressed microspores, the further development of the intine, after transfer to non-stress condition, is deviant.

The stress pretreatments caused also a structural reorganization of the cytoplasm of wheat microspores. The large central vacuole did not disappear as it would during normal pollen development, but become fragmented due to the formation of cytoplasmic strands that pass through the vacuole and connected the cytoplasmic pocket (phragmosome) which surrounds the nucleus with subcortical cytoplasm (Figs. 2.A., B., C., D.). The fragmentation of vacuole was also observed in other species such as tobacco (Garrido *et al.*, 1995; Touraev *et al.*, 1997), brassica (Zaki and Dickinson, 1990) and seem to be a characteristic of the embryogenic state of microspores. These morphological changes may also be the response of microspores to stress. The precence of many nucleopore (Fig. 2.E.) are indicative of the existence of communication between the cytoplasm which had programed sporophytically and the nucleus.

Development of embryogenic wheat microspores in embryogenesis medium A2

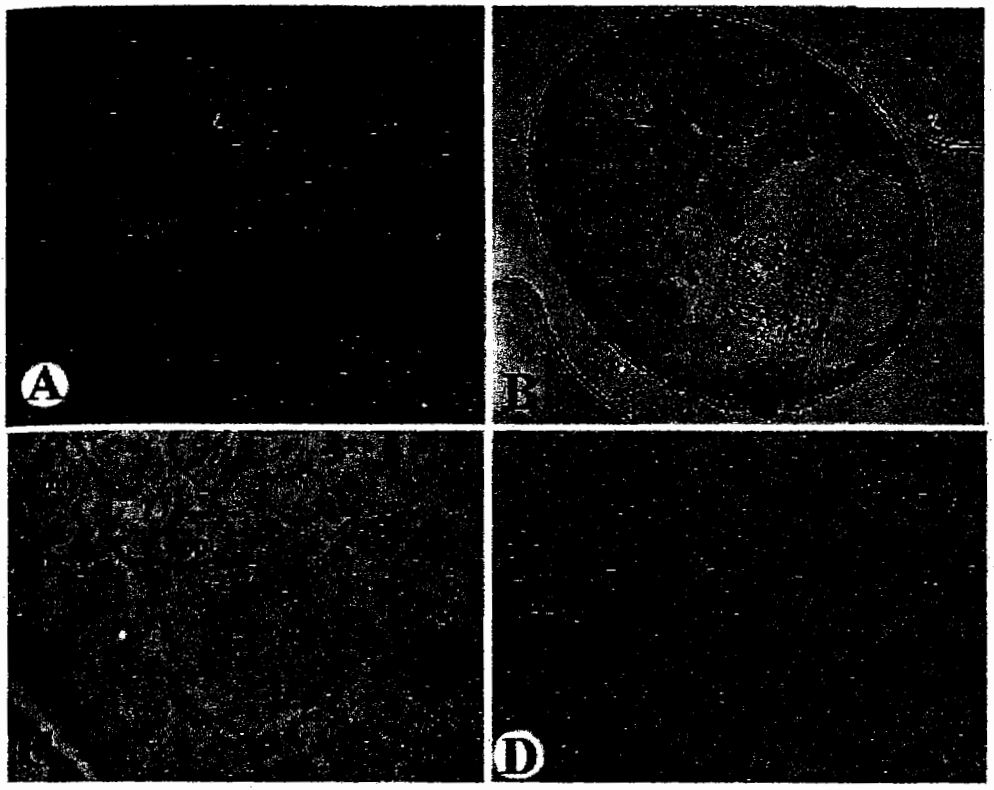
Stressed microspores were isolated from the anthers and cultured in medium A2 in presence of 6 immature ovaries at

25°C. Cytological analysis of microspores stained with DAPI 2 days after culture showed that approximately 22% of microspores divides symmetrically, forming two vegetative-like nuclei of similar size (Table 1., Figs. 3.A., B.). This developmental pathway is described in the literature as the B pathway (Sunderland *et al.*, 1979). In 3% of the binucleate pollen with vegetative and generative cells, the vegetative nucleus is also divided symmetrically. This data indicate that in total 25% of the population of micro-spores and binucleate pollen divided symmetrically within 48 hours. Notably, all divisions observed are symmetrical. The population of unicellular microspores decreased from 84% to 70% (Table 1). The symmetric division of the microspores is probably derived from microspores with a nucleus arrested in G1 phase of the cell cycle, while the division of vegetative nucleus in binucleate pollen may be the result of the derepression of cell cycle of vegetative nucleus during stress pretreatments which had passed through the regular asymmetric first haploid mitosis during stress pretreatment and which had been in the G2 phase at the time of excision. Similar results have been reported in tobacco (Zarsky *et al.*, 1995).

Cell plate formation between two identical nuclei was not observed. The wall which separated these nuclei had an electron density similar to the intine (Fig. 3.D.). In embryogenic microspores, during *in vitro* culture, intine continue to increse in thickness. In several areas the intine formed centripetally oriented protrusion inside the cytoplasm (Fig. 3.C.). Probably, this is the first step of the intine participation in cell wall formation of dividing embryogenic microspores. In barley, cell wall formation in multinuclear pollen grain also initiated by the intine (Idzikowska and Mlodzianowski, 1979). Huang (1986) proposed that in wheat pollen cultured *in vitro*, cell wall formation accompanying pollen embryogenesis is either via cell plate or wall ingrowth.

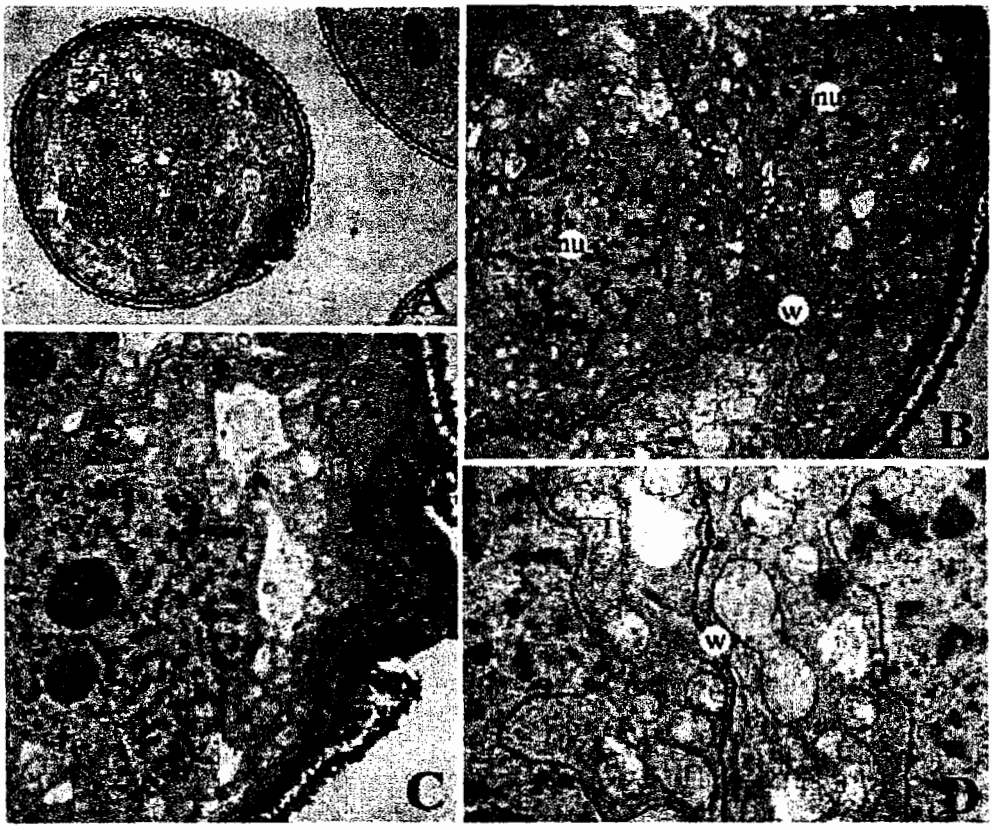
The cytoplasm of embryogenic microspores cultured in medium A2 proliferated with the progression of culture periods and become rich in organelles (Fig. 3.C.). An increase in endoplasmic reticulum and dictyosome seems to be related to the role of these organelles in the cell wall formation while budding of the condensed mitochondria with cristae reflects the metabolic activity of the cytoplasm. The appearance of lipids droplets and proliferation of all cytoplasmic contains are the features common to the cells of meristematic tissue.

Six days after culture, the frequency of uninucleate, binucleate and trinucleate pollen grain decreased to 9%, 21% and 3 %, respectively. In addition, approximately 46% of the population consisted of multicellular structures (Table 1.).



Figures 3A - D: Development of embryogenic wheat microspores after 2 days in embryogenesis medium A2

A. Symmetric division of the embryogenic microspores after stained with DAPI (X 1000).
B. Ultrastructure of symmetrical division of an embryogenic microspore. The wall separating the two identical cells is in the same axis as the line from pore to nucleus (nu) in the microspore (X 1100).
C. Dictyosome (ds), endoplasmic reticulum (er), lipid droplets (ld), mitochondria (mt) and proplastid (ppl) in the cytoplasm of embryogenic microspore. Proplastids are much smaller than the mitochondria, protrusion of the intine (in) wall is also seen (X 12000).
D. The wall which separates two daughter cells, has the same electron density as the intine (in, X 7000)



Figures 4A - C: Development of embryogenic microspores after 6 days in embryogenesis medium A2.

A – C. Multicellular pollen with distinct wall (w) separating each cell (A: X 1100; B: X 3000; C: X 3000.

D. Distinct wall (w) without plasmodesmatal connection between two daughter cells (X 12.000).

Cellularization of multinuclear structures are observed in some (Fig. 4.) of these structures.

CONCLUSION

In this report we present cytological and ultrastructural features of the initiation of embryogenesis in isolated wheat microspore cultures: a). to identify and characterize an embryogenic microspore, and b). to characterize ultrastructural and cytological changes during the initiation of

embryogenesis.

We show here that the subjection of microspores to stress pretreatments such as cold, heat or starvation cause morphological changes in the microspores. The majority of stressed microspores became "star-like" which is characterized by the fragmentation of the vacuole and location of the nucleus close to the center inside the cytoplasmic pocket (phragmosome). First sporophytic division is symmetrical. Although we have shown many changes during the first days of initiation of sporophytic division, the question remains whether these changes are specific for the embryogenesis pathway or the result of the response of microspores to external stresses.

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